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ANTHER AND POLLEN DEVELOPMENT IN PEAS AND LENTILS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Anther and Pollen Development in Peas and Lentils" submitted by Julie Anne Biddle in partial fulfilment of the requirements for the degree of Master of Science in Structural Botany.

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ABSTRACT

After conventional fixation procedures and embedding in low viscosity resin, anthers were studied using light and electron microscopy. Wall development is of the dicotyledonous type. Endothecial cells develop extensive thickenings which do not react readily to standard histochemical tests including Hydroxylamine-ferric-chloride (Reeve 1959), PAS test (adapted from McManus 1948), IKI - H_2SO_4 (Jensen 1962), Schiff's (McLean and Cook 1941) and Sudan Black B (Bronner 1975). Various stages in the development of the secretory tapetum have been followed. It starts to senesce around the time of microspore mitosis, but does not finally degenerate until pollen grains are at the vacuolate stage. Sporogenous tissue undergoes one mitosis before meiosis. Entry of pollen mother cells (PMC's) into meiosis is indicated by formation of a prominent polar nucleolar cap. Cytomictic channels form between dividing PMC's in early prophase I. The middle lamella breaks down between the meiocytes, and an unusual type of wall formation is seen. The quartets are surrounded by an extensive callose special wall within which the primexine starts to form. Callose dissolution begins at this stage and is centripetal. Once released from the callose wall, development of tectate exine continues; pollen cytoplasm becomes vacuolate. Minor variations occur between garden pea and lentil, but the general sequence is comparable. These results extent prior information on and confirm existence of relative homogeneity of anther development in the Leguminosae.

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TABLE OF CONTENTS

CHAPTER	PAGE
1. Introduction	1
2. Materials and Methods	3
3. Results	5
3.1. Stage A: Primary sporogenous tissue	5
3.1.1. Wall development	5
3.1.2. Sporogenous tissue development	6
3.2. Stage B: Interphase before meiosis I	8
3.2.1. Wall development	8
3.2.2. Tapetum	8
3.2.3. Pollen mother cells	9
3.3. Stage C: Meiosis	10
3.3.1. Wall development	10
3.3.2. Pollen mother cells	10
3.4. Stage D: Quartet	13
3.4.1. Wall development	13
3.4.2. Quartet	14
3.5. Stage E: Microspore mitosis	18
3.5.1. Wall Development	18
3.5.2. Microspores	18
3.6. Stage F: Pollen grains begin to vacuolate	19
3.6.1. Wall development	19
3.6.2. Pollen grains	20

CHAPTER PAGE

3.7. Stage G: Vacuolate pollen grains 21

3.7.1. Wall development 21

3.7.2. Pollen grains 21

3.8. Stage H: Mature pollen grains 22

3.8.1. Wall development 22

3.8.2. Pollen grains 22

4. Discussion 25

4.1. Callose 28

4.1.1. Deposition 28

4.2.2. Dissolution 28

4.2. Tapetum 29

4.3. Angiosperm primexine development 35

4.4. Angiosperm exine development 37

4.5. Gymnosperm exine development 38

4.6. Mature pollen cytoplasm 39

4.6.1. Vegetative cell 39

4.6.2. Generative cell 41

4.7. Endothecium 42

4.8. Conclusions 46

5. Bibliography 59

Appendix I: Fixation and embedding schedule 67

Appendix II: Staining procedures 69

LIST OF TABLES

TABLE PAGE

1. Summary of histochemical testing of the endothecium 23

LIST OF FIGURES

FIGURE		PAGE
1.	Light micrograph of *Pisum* showing 2 daughter cells of primary parietal cell division	48
2.	Light micrograph of *Pisum* loculus with pre-meiotic interphase pollen mother cells	48
3.	Light micrograph of *Pisum* anther with 3-4 layered wall	48
4.	Light micrograph of *Pisum* with 2-layered wall	48
5.	Light micrograph of *Lens* anther with 3-layered wall	48
6.	Electron micrograph of *Pisum* pre-mitotic interphase sporogenous cell	49
7.	Electron micrograph of *Pisum* pollen mother cell at pre-meiotic interphase	49
8.	Electron micrograph of *Pisum* 4-layered anther wall	49
9.	Electron micrograph of *Pisum* tapetal cell	49
10.	Electron micrograph of *Lens* pollen mother cell at prophase of meiosis I	50
11.	Light micrograph of *Lens* pollen mother cell at pachytene of meiosis I	50
12.	Light micrograph of *Lens* - anaphase of meiosis I	50
13.	Electron micrograph of *Pisum* pollen mother cell at diplotene of prophase I	50
14.	Electron micrograph showing unusual primary wall formation occurring between meiocytes of *Pisum*	50
15.	Electron micrograph of *Lens* cytomictic channel	50
16.	Light micrograph of *Lens* anther loculus after Sudan black b staining	51
17.	Light micrograph of *Pisum* quartets in which the microspores are tetrahedrally arranged	51

FIGURE PAGE

18. Light micrograph of Pisum quartets 51

19. Light micrograph of Lens quartets in anther loculus . . . 51

20. Fluorescence micrograph of Pisum quartets illustrated in Fig. 18 51

21. Fluorescence micrograph of Lens anther loculus illustrated in Fig. 19 51

22. Electron micrograph of Pisum microspore just prior to complete centripetal callose dissolution 52

23. Electron micrograph of Pisum aperture 52

24. Electron micrograph of Lens microspore, showing centripetal dissolution of the callose special wall 52

25. Electron micrograph of Pisum tapetum just prior to dissolution of the callose special wall 52

26. Electron micrograph of Lens epidermal cell 52

27. Electron micrograph of Pisum tapetum in which the radial cell wall has started to break down 52

28. Light micrograph of Pisum microspores 53

29. Electron micrograph of Pisum in the early stages of primexine formation 53

30. Electron micrograph of Pisum electron-dense probaculae 53

31. Electron micrograph of Pisum probaculae 53

32. Electron micrograph of Pisum microspore just prior to mitosis 54

33. Electron micrograph of Pisum tapetal cell 54

34. Electron micrograph of Lens endothecium which has bulbous wall thickenings 54

35. Electron micrograph of Pisum tapetum 54

36. Electron micrograph of Lens tapetum after disappearance of the nucleus 55

37. Electron micrograph of Pisum tapetum 55

FIGURE PAGE

38. Electron micrograph of Lens tapetum with largely intact plasmalemma 55

39. Electron micrograph of Pisum tapetum inner tangential wall 55

40. Light micrograph of Pisum vacuolate pollen grains 56

41. Electron micrograph of Pisum generative cell 56

42. Electron micrograph of Lens in which transfer of tapetal and middle layer remains is occurring 56

43. Electron micrograph of electron-dense Pisum tapetal and middle layer remains 56

44. Light micrograph of Lens anther loculus with mature pollen grains 57

45. Electron micrograph of Pisum exine prior to intine formation 57

46. Light micrograph of Lens mature pollen after Sudan black b staining 57

47. Electron micrograph of Lens endothecial cell fibrous wall thickening 57

48. Electron micrograph of Lens mature pollen cytoplasm . . . 58

49. Electron micrograph of Pisum mature pollen cytoplasm in aperture 58

50. Electron micrograph of Lens mature pollen cytoplasm - inner zone 58

51. Electron micrograph of Lens mature pollen exine 58

52. Dicotyledonous anther wall development 7

53. Monocotyledonous anther wall development 7

54. Pollen grain wall terminology 16

CHAPTER 1

INTRODUCTION

Since De Candolle in 1825 first recognized the tribe Vicieae, it has remained unaltered except for the inclusion by some authors of the genus Abrus. Recently it has been suggested on the basis of pollen morphology and other taxonomic characters that Cicer should be removed from the Vicieae and placed in its own monogeneric tribe Cicereae Alef. (Kupicha 1977; Clarke and Kupicha 1976).

The Vicieae tribe includes a number of economically important members e.g. Vicia faba - broad bean, Pisum sativum - garden pea, Lens culinare - cultivated lentil, and Cicer arietinum - chick pea (Clarke and Kupicha 1976; Kupicha 1977). Lens, on the basis of archeological evidence, is among the oldest domesticated plants. Cultivation of this crop is thought to have begun around 7000-6000 BC in the Middle East (Zohary 1972).

There have been a number of studies in the Leguminosae on the development of anthers and pollen (Buss et al. 1969; Hindmarsh 1964; Latter 1926). To my knowledge, the study of pollen wall development in Parkinsonia by Larson and Lewis (1962) is the only electron microscope investigation carried out in the Leguminosae.

Chromosome morphology of Pisum, one of the members of the Vicieae has been extensively investigated (Blixt 1958a, 1958b, 1959; Gottschalk and Klein 1976; Gottschalk and Milutinovic 1973). However only two reports in the literature deal with microsporogenesis in

either Pisum (Cooper 1938) or Lens (Shukla 1954), both of which are cleistogamous (Wilson 1972). As chromosome morphology in both mitosis and meiosis had been well-studied in Pisum I decided to concentrate my investigations of anther and pollen development in it and Lens in different areas. My main emphasis then in the correlated light and electron microscope approach has been concentrated on pollen wall formation, development of the tapetum, the endothecium, pollen cytoplasm organelles and the nature of the generative cell.

For organizational purposes I have arbitrarily divided stages in anther and pollen development into 8 categories. Within each of these categories, development of sporogenous tissue, tapetum, and other anther wall layers is correlated.

CHAPTER 2

MATERIALS AND METHODS

Pisum sativum L. var lincoln and Lens culinare Medic. 1787 (syn. Lens esculenta Moench 1794) seeds were grown at 24C with 16 hours of light and a light intensity of no more than 32,000 Lux. Plants required five to eight weeks to flower under these conditions.

To determine their developmental stage Pisum anthers were initially sampled from buds on a length and weight basis. This proved to be unreliable and was even less successful in Lens. A better procedure was to make a fresh anther squash preparation from a bud and if it was at the desired stage to fix the remaining anthers from that bud. Usually all anthers in the same bud were at the same developmental stage.

Anthers were fixed in 3% glutaraldehyde in phosphate buffer at pH 6.8 (Jensen 1962) for 3 hours, washed in the same buffer for an hour and postfixed in 2% osmium tetroxide in the same buffer for 2 hours. In some cases no buffer wash was used after the second fixative; no observable undesirable effects were found even at the electron microscope level. This agrees with work on some animal tissues (Ockelford 1975). The material was dehydrated in an ethanol series and embedded in low-viscosity resin (Spurr 1969) - for further details of procedures used see Appendix I. Blocks were sectioned on a Reichert OM U2 Ultramicrotome and then viewed under either a Zeiss photomicroscope or a Philips EM 200, EM 201, EM 300 electron microscope.

Light microscope sections were routinely stained with either aniline blue black (cf Appendix II) or toluidine blue (cf Appendix II). Tissue for electron microscopy was stained with uranyl acetate (cf Appendix II) and then with lead citrate (Venable and Coggeshall 1965).

Various histochemical procedures were performed for light microscopy: aniline blue fluorescence (cf Appendix II) for callose; azure B (Hoefert 1968) and basic fuchsin (Huber et al. 1968) for RNA; hydroxylamine-ferric chloride (Reeve 1959) and ruthenium red (Johansen 1940 and Sterling 1970) for pectins; periodic acid - Schiff's (cf Appendix II) for insoluble polysaccharides; IKI (cf Appendix II) for starch; zinc-chlor-iodide (Rawlins and Takahashi 1952) and IKI - H_2SO_4 (Jensen 1962) for cellulose; phloroglucinol-HCl (Jensen 1962), chlorine-sulphite (cf Appendix II), Schiff's reagent (McLean and Cook 1941) and Maule reaction (De Fossard 1969) for lignin; Sudan black b (Bronner 1975) for lipids; aceto-carmine (cf Appendix II) and acetic-orcein (La Cour 1941) for chromosomes.

CHAPTER 3

RESULTS

3.1. Stage A: Primary sporogenous tissue

3.1.1. Wall development

In the youngest stage observed two wall layers are present; the outer epidermal layer and the inner primary parietal layer (Fig. 4).

The epidermal tissue divides only anticlinally while the parietal divides both periclinally and anticlinally. During periclinal division the parietal layer gives rise to secondary parietal tissue. Two products of such a division can be seen in Fig. 1. Both cells have prominent spherical nuclei and their chromatin has not yet returned to the resting state.

The anther wall is now 3-layered and the individual layers show a closer morphological resemblance to each other than to the sporogenous tissue (Fig. 5). Epidermal cells have become more vacuolate during anther wall development and usually contain a large vacuole. Within epidermal cell vacuoles in Lens, crystals of unknown chemical nature often occur. The other 2 layers more closely resemble each other than the epidermis. Although they are vacuolate they often have a number of small vacuoles rather than a single large one. Sudan black b staining indicates that there is a small amount of lipid in each wall layer.

In Lens amyloplasts are a conspicuous feature of the cytoplasm of each cell layer; however, no starch grains have been found in Pisum

although plastids having 2 thylakoid stacks do occur at this stage.

3.1.2. Sporogenous tissue development

During this time the primary sporogenous tissue undergoes one mitotic division, probably the only pre-meiotic division, to form the secondary sporogenous tissue. Fig. 5 shows anaphase of this division in one sporogenous cell in Lens. The other cells in Fig. 5 have pre-mitotic interphase nuclei. Within the nucleus condensed chromatin and a large spherical electron-dense nucleolus are visible; slightly less electron-dense areas may occur within nucleoli (Fig. 6). From 1-4 micronucleoli may be formed in a single nucleus (Figs. 5 , 6).

The cytoplasm of these secondary sporogenous or pollen mother cells is quite dense with many ribosomes and only few, small vacuoles. Many mitochondria with poorly developed cristae and some rather electron-dense plastids which show little internal differentiation also occur. Some apparently active dictyosomes and fine strands of rough endoplasmic reticulum (RER) are found scattered through the cytoplasm. In some cells concentric membrane lamellae and "double membrane-bound inclusions" are present. Individual pollen mother cells are interconnected by plasmodesmata (Fig. 6).

Fig. 52

DICOTYLEDONOUS ANTHER WALL DEVELOPMENT

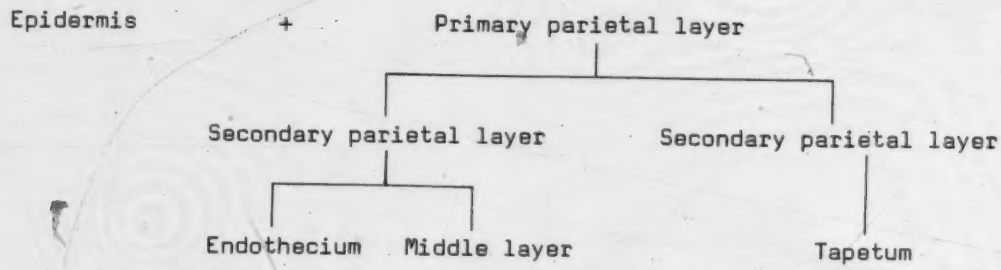
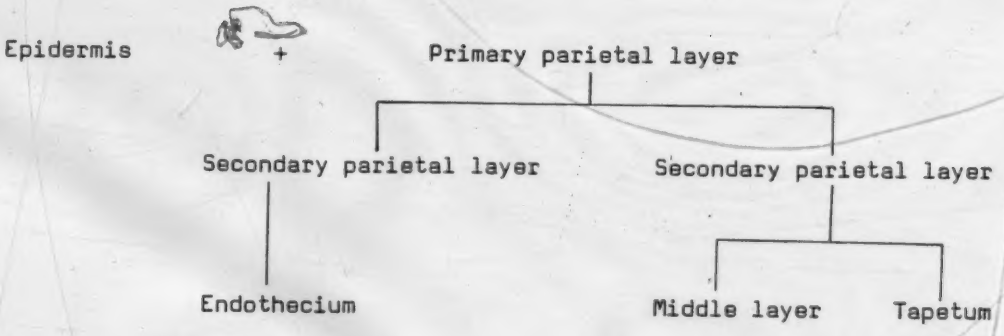


Fig. 53



3.2. Stage B: Interphase before meiosis I.

3.2.1. Wall development

The secondary parietal cells nearest the epidermis divide periclinally to produce externally the endothecium and internally the middle layer (Fig. 52). Fig. 3 illustrates anaphase of such a division in Pisum. In dicotyledonous anther wall development, the tapetum differentiates directly from a secondary parietal cell nearest the sporogenous tissue (Fig. 52). I can report one instance where the tapetum develops as a result of division of secondary parietal tissue which would correspond to the monocotyledonous type of wall formation; I regard this as an unusual variation (Fig. 53). Usually the tapetum consists of only a single cell layer; however, occasional supernumerary divisions are observed.

Within epidermal, endothecial and middle layer cells the plastids begin to develop starch grains in Pisum. Vacuolar lipid deposits also occur in some epidermal cells of both species (Fig. 8).

Plasmodesmatal connections have been found between all anther wall layers except adjacent epidermal cells. None have been seen between the tapetum and the pollen mother cells.

The middle layer cells with prominent interphase nuclei contain plastids with few lamellae, a small amount of lipid and starch; apparently active dictyosomes and inflated strands of primarily rough endoplasmic reticulum (RER) are closer to the tapetum than to the other anther wall layers.

3.2.2. Tapetum

When the secondary parietal layer divides to form the last 2 layers of the anther wall, the tapetum begins to differentiate. At this time it morphologically resembles the pollen mother cells (Fig. 3).

However as differentiation proceeds this resemblance diminishes (Fig. 2).

The tapetal cell has a prominent interphase nucleus which usually contains 1 nucleolus, rarely 2. These nucleoli are spherical and electron-dense, although at times they contain areas of lower electron density. Small vacuoles occurring around the nucleus are clearly visible with light microscopy (Fig. 2). The plasmalemma between tapetal cells often becomes highly convoluted and occasional cytomictic channels may form. The plastids have few lamellae and small amounts of lipid and starch. The dictyosomes appear to be actively producing vesicles (Fig. 9). Inflated strands of primarily RER ramify throughout the cell.

3.2.3. Pollen mother cells

The most readily discernible change in these cells at this stage is the production of numerous small vacuoles (Fig. 2). The interphase nucleus appears to have large dilatations between outer and inner envelopes, primarily between pores. Dilated RER cisternae are scattered through the cytoplasm and plastids appear to be fewer in number (Fig. 7).

3.3 Stage C: Meiosis

3.3.1. Wall development

With exception of the tapetum few changes occur in the anther wall at this stage. Sudan black b staining reveals the presence of some lipid in tapetal cytoplasm. In Lens starch grains occur in some plastids (Fig. 10), while in Pisum these are no longer present. In Lens radial tapetal cell walls are not convoluted and although there are plasmodesmata, no cytomictic channels have formed; Pisum exhibits both convoluted walls and cytomictic channels between tapetal cells. There are fewer ribosomes resulting in lighter staining of the cell cytoplasm in Lens. The RER is in fine hair-like strands in Lens and tapetal crystals of unknown composition are seen in each tapetal cell (Fig. 10).

3.3.2. Pollen mother cells

MEIOSIS

The pollen mother cells (PMC) now pass through the various stages of meiosis. The earliest stage seen is pachytene of prophase I in which the chromosomes have an elongate, stranded appearance. The nucleolus has assumed a polar position but it is more kidney-shaped at this time. The pars granulosa occupies the narrow peripheral region of the nucleolus and the lighter pars amorpha the inner part (Fig. 11). Azure B staining indicates the presence of RNA in both parts of the nucleolus and in the 1 or 2 micronucleoli, which may form. During late pachytene the nucleolus assumes a crescentic shape and the chromosomes become more contracted. Further chromosome contraction occurs during diplotene (Fig. 13) and diakinesis. By metaphase the nucleolus has disappeared and the darkly staining chromosomes have assumed a more

central position. At anaphase I they begin to move towards opposite poles (Fig. 12). The end product of meiosis I is a binucleate cell; stages of meiosis II have not been observed. A trinucleate cell has been found, but cytokinesis in it has not been seen.

By pachytene large cytomictic channels, which may be observable even with light microscopy, form between meiocytes (Figs. 11, 15). Under the electron microscope organelles have been seen to pass through them. Most plasmodesmatal connections are severed at this time; sometimes a remnant of a severed one may be sectioned (Fig. 13).

In Pisum, the loculus may expand substantially during this period so that individual meiocytes are not in close contact.

POLLEN MOTHER CELL CYTOPLASM

Sudan black b staining indicates that there are small amounts of lipid in the cytoplasm. Plastids are now apparent and mitochondria have better-developed cristae. The RER is in fine hair-like strands extending throughout the cell as before. Active-appearing dictyosomes have been seen in Lens but no dictyosomes were observed in Pisum. A few small vacuoles occur in the cytoplasm of each species. Normally the nucleolus is uniformly electron-dense but light areas may sometimes form within it.

UNUSUAL WALL FORMATION

An unusual wall formation has been observed in Pisum (Figs. 13, 14). The middle lamella has apparently broken down between the individual meiocytes but their electron-dense cell walls are still present. The cell walls have a fine fibrillar appearance. Inside the cell wall is a less electron-dense fibrillar layer which varies in thickness (Figs. 13, 14). I do not think that this marks the beginning of callose

deposition because callose is amorphous and also because the aniline blue fluorescence used on this wall has yielded negative results. This wall elaboration has not been observed in Lens. Karas and McCully (1973) report a similar type of wall in the endodermis of Zea mays. In stylar transmitting tissue of Petunia, Capsella, Vitis, Lythrum (Sassen 1974), the middle lamella between the cells breaks down and is replaced by an intercellular substance. A number of instances of similar autolysis of portions of unlignified walls are reported by O'Brien and Carr (1970). I do not know whether this represents wall autolysis in Pisum. This wall formation has not been seen before in dividing pollen mother cells.

3.4. Stage D: Quartet

3.4.1. Wall development

The endothecium does not undergo significant changes during this period.

EPIDERMIS AND MIDDLE LAYER

In Pisum few of the epidermal cells are nucleated and senescence seems to have progressed further than in Lens where most of the cells remain nucleated. Another feature distinctive of Lens is the presence of electron-dense vacuolar inclusions (Fig. 26). Their reaction to Sudan black b is sporadic; this suggests that they may not be entirely lipid in nature (Fig. 16). Larger inclusions occur in the central portion of the vacuole and smaller ones peripherally. The smaller inclusions are also seen in middle layer cells; the middle layer cells are nucleated, vacuolate, have diffuse cytoplasm, and fine hair-like strands of RER.

TAPETUM

Two different stages in development of the tapetum are reported: younger, when the quartets are still encased in callose; older, when final dissolution of the callose special wall occurs.

In the earlier stage the cell wall between the tapetal cells starts to break down. The plasmalemma remains intact and is convoluted in both species. Cytomictic channels have not been seen but the tapetal cells are still connected by plasmodesmata. There are usually large vacuoles on both sides of the nucleus. They may have electron-dense inclusions within them. Active-appearing dictyosomes are found in the tapetal cytoplasm (Fig. 27). In Lens the tapetal crystals present during meiosis have disappeared. In Pisum occasional binucleate tapetal cells have been noted.

In the older stage tapetal cells extend further into the loculus (Fig. 28). Part of the radial cell wall and of the inner tangential wall (ITW) has broken down. The plasmalemma is highly convoluted both radially and on the inner tangential face (Fig. 25). Cytomictic channels, which replace the existing plasmodesmata have formed between the cells. Increased conspicuity of plastids, including possession of more electron-dense inclusions, may be due to a diminished overall cytoplasmic density. The rough or smooth endoplasmic reticulum, which has a rope-like appearance has proliferated and now ramifies throughout the cell often with a number of strands following the same path (Fig. 25).

3.4.2. Quartet

The cell wall around the quartets does not react positively to zinc-chlor-iodide test for cellulose but it does give a strong reaction with the ruthenium red test for pectins.

The quartet of microspores may show a tetrahedral or apparent decussate arrangement. Tetrahedral is the most common in Pisum; in Lens it is the only arrangement observed (Figs. 17, 18, 20). The microspores are embedded in extensive callose special walls which react positively to the aniline blue fluorescence method (Figs. 18-21). Cytomictic channels have been severed by this time and each microspore is now isolated from its neighbours by the callose special wall. In Lens the individual quartets lie close together. However in Pisum the loculus may so enlarge that the individual quartets are separated from each other.

A change in microspore shape may occur during callose dissolution and deposition of primexine. This is particularly apparent in

those of the tetrahedral quartets. Initially they tend to be "triangular"; however when older they are rounded in transection (TS) and elongate in longitudinal section (LS) (Fig. 28).

MICROSPORE CYTOPLASM

Each microspore has a prominent interphase nucleus with a single spherical nucleolus; however, up to four nucleoli have been seen in the one nucleus (Figs. 17, 28). The cytoplasm is largely non-vacuolate with only a few small vacuoles occurring in Lens; some microspore vacuoles have electron-dense inclusions. Microspore vacuoles in Pisum are more numerous and occupy the inner cytoplasm around the nucleus (Fig. 22). Lipid deposits have also been verified with Sudan black b staining (Fig. 16). Apparently active dictyosomes occur throughout the cytoplasm as do irregularly-shaped electron-dense plastids. There are numerous mitochondria and fine strands of RER.

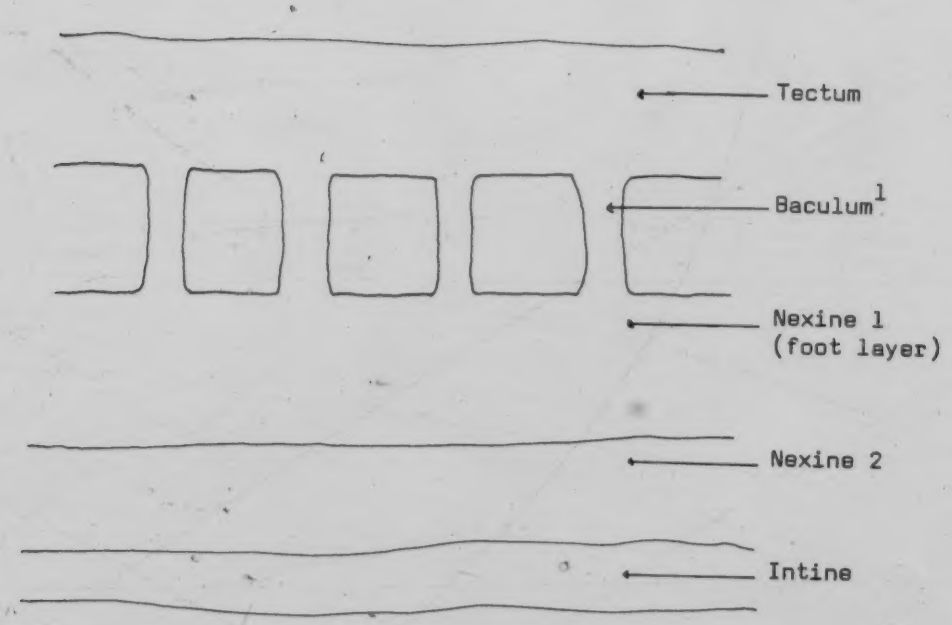
CALLOSE DISSOLUTION

The dissolution of callose surrounding microspores is generally centripetal (Figs. 21, 22). In tetrahedral quartets this dissolution begins at the corners of the tetrahedron. Channels extend into the callose wall. The callose lying closest to the microspore plasmalemma is more electron-dense than the rest. Whether this is a phenomenon of deposition or of dissolution is unknown. Electron-dense, almost fibrillar material resembling that in the locular cytoplasm occurs in the channels (Fig. 24). I have seen one case where callose dissolution proceeded both centripetally and centrifugally. Where the quartets abut the tapetum, electron-dense remnants of severed plasmodesmata may occasionally project into the channels.

Fig. 54

POLLEN GRAIN WALL TERMINOLOGY

Erdtman (1969)



[1]Prior to callose special wall dissolution baculae are known as probaculae.

PRIMEXINE DEVELOPMENT

Before primexine development begins the microspore plasmalemma may be somewhat undulate. A fibrillar layer of primexine matrix forms over the microspore surface except in future apertural regions (Fig. 29). It is in this layer that probaculae are laid down. They first begin to form on adjacent microspore surfaces. Next probaculae are laid down discontinuously around the microspore surface (cf Fig. 54 for a summary of pollen grain terminology used in the text). By this time the callose special wall has begun to dissolve.

The probaculae are first seen as small, almost square blocks radiating from the microspore plasmalemma (Fig. 29). They are more electron-dense than the primexine matrix in which they are deposited; the underlying plasmalemma is undulate. The probaculae continue to enlarge, becoming almost wedge-shaped (Fig. 31). Not only undulation of the plasmalemma but more elaborate extensions of it have been seen in the early stages of probaculum deposition (Fig. 24). These membranous protrusions may join with the base of the probaculum and/or bud off to form distinct vesicles below the primexine. Next the probaculae become more electron-dense and initial stages of nexine 1 formation are now discernible (Fig. 30). Early nexine 1 formation is almost concomitant with the final stages of callose dissolution and possible sporopollenin deposition. The laying down of the tectum also begins at this time (Figs. 22, 30). Fragments of exine may cover the aperture which is made up of fibrillar material (Fig. 23).

3.5. Stage E: Microspore Mitosis

3.5.1. Wall development

No significant changes occur in the anther wall during this period. Occasionally in Lens, crystals have been seen in the vacuoles of the tapetal cells. Whether these represent a re-deposition of crystalline material since the quartet stage or crystals which did not break down at all is not known.

3.5.2. Microspores

This is the time period just prior to microspore mitosis to form a binucleate pollen grain and during it the microspore cytoplasm appears the same as in the quartet stage. The callose special wall has completely dissolved, and single microspores now occur in the anther loculus. Further sporopollenin deposition has taken place resulting in greater electron-density of the nearly totally tectate exine. Formation of the narrow nexine 1 layer is complete beneath the baculae; it is thickest at the aperture margins, not covering the apertures but extending only up to their sides (Fig. 32). The aperture is covered with baculae and tectum.

3.6. Stage F: Pollen grains begin to vacuolate

3.6.1. Wall development

ENDOTHECIUM

The endothecium now develops extensive wall thickenings (Fig. 34). Endothecial cell thickenings do not react to Sudan black b staining and also contain little fibrillar material. The plastids now lack starch which had been a conspicuous feature until this stage.

TAPETUM

The tapetum proceeds through a series of senescent stages. At first each tapetal cell has a prominent interphase nucleus with an electron-dense spherical nucleolus; a small electron-dense micronucleolus may form. A large vacuole and lipid-containing plastids both with and without starch grains are found in the cytoplasm. Hair-like strands of RER ramify throughout the cell (Fig. 33). Next, electron-dense lipid droplets are found along the radial walls and there is a proliferation of the RER in the cytoplasm. Small vacuoles have developed (Fig. 35).

The nucleus starts to break down and the cytoplasm is more vacuolate. Plastids no longer contain starch and some of them begin to break down. Lipid droplets lie around the circumference of degenerating mitochondria and much RER has disappeared (Fig. 37). The ITW is convoluted with most of the plasmalemma intact. Electron-dense lipid droplets and "spiny" vesicles (Esau and Gill 1970; Newcomb 1967) are found near the plasmalemma. Small fragments of cytoplasm break off into the loculus (Fig. 39).

After disappearance of the nucleus the cytoplasm is more diffuse; most of the contents of mitochondria have disappeared but peripheral

electron-dense lipid droplets are still present. Rough endoplasmic reticulum strands are aligned along the outer tangential wall (OTW) and ITW (Fig. 36). Although a further decrease in cytoplasmic contents occurs as is characteristic of secretory tapeta the plasmalemma remains intact (Fig. 38). Once, in Pisum I observed breakdown of the walls of nucleated tapetal cells and subsequent release of their contents into the loculus.

3.6.2. Pollen grains

Stages in microspore mitosis have not been observed. The pollen grains are more vacuolate and the nexine 2 layer has formed. It is thickest on the aperture margins. The exine stains positively with Sudan black b.

3.7. Stage G: Vacuolate pollen grains

3.7.1. Wall development

TAPETUM AND MIDDLE LAYER

Sudan black b staining shows that the tapetal and middle layer remains are predominately lipid. They are electron-dense (Fig. 43) and are sloughed off into the loculus (Fig. 40). Electron-dense remains are deposited both on the pollen grain tectum and interbacularly (Fig. 42).

3.7.2. Pollen grains

The pollen grain cytoplasm is vacuolate. The vegetative nucleus has a large, electron-dense nucleolus which usually has less electron-dense areas within it (Fig. 40). The generative cell nucleus is at interphase and contains a small spherical electron-dense nucleolus (Fig. 41). Its cytoplasm has active-appearing dictyosomes, mitochondria, a vacuole and fine strands of RER (Fig. 41). Formation of the narrow intine is complete by now. On 2 occasions in which intine formation had not taken place, either vesicles with electron-dense peripheral deposits or electron-dense deposits alone are seen between the plasmalemma and nexine 2 layer (Fig. 45).

3.8. Stage H: Mature pollen grains

3.8.1. Wall development

ENDOTHECIUM

In Pisum the fibrous endothecial thickenings (Fig. 47) did not react to Sudan black b for lipids; hydroxylamine ferric chloride for pectins; phloroglucinol-HCl, Schiff's reagent, chlorine-sulphite test, Maule reaction and UV light for lignin. They reacted positively with IKI-H_2SO_4 for lignin; faintly both with ruthenium red for pectins and zinc-chlor-iodide for cellulose. In Lens results were the same except for a faint reaction to hydroxylamine ferric chloride for pectins and a positive reaction to UV light for lignin. See Table 1 for a summary of histochemical testing of the endothecium. Endothecial cell cytoplasm is now senescing. It has become diffuse, vacuolate and the plastids are breaking down.

TAPETUM AND MIDDLE LAYER

Both these layers have disappeared (Fig. 44) except for a few remains which may be visible with the electron microscope.

3.8.2. Pollen grains

The fibrous intine (Fig. 51) reacts strongly to ruthenium red and hydroxylamine ferric chloride tests for pectins. The exine no longer reacts positively to Sudan black b. Interbacular deposits react positively to Sudan black b as do inner or core cytoplasm lipid droplets. The stranded appearance of some of the Sudan black b positive material may represent the central portion of the RER bilayer in Lens (Fig. 46). Pisum cytoplasm is not as clearly zonated as that of Lens. In the outer zone mitochondria with well-developed cristae and dictyosomes are numerous. There are also fine strands of RER. In the

TABLE 1

SUMMARY OF HISTOCHEMICAL TESTING OF THE ENDOTHECIUM

		Pisum	Lens
Pectins	Ruthenium Red	FR	FR
	Hydroxylamine ferric chloride	NR	FR
Lignin	Schiff's reagent	NR	NR
	Maule reaction	NR	NR
	Chlorine-sulphite	NR	NR
	Phloroglucinol-HCl	NR	NR
	Fluorescence microscopy	NR	R
Cellulose	IKI-H_2SO_4	R for lignin	R for lignin
	Zinc-chlor-iodide	FR	FR
	Polarizing microscopy	R	R
Lipid	Sudan Black B	NR	NR

FR - faint reaction

NR - no reaction

R - positive reaction

central region vacuoles and electron-dense deposits predominate (Fig. 49). In the outer zone of Lens cytoplasm there are mitochondria, numerous vesicles and dicytosomes (Fig. 48). The inner zone near the lobed vegetative nucleus has many vacuoles and lipid bodies. Both vacuoles and lipid bodies are often surrounded by RER with presumed lipid material sandwiched between the 2 strands. Other portions of the RER bilayer extend throughout the cytoplasm (Fig. 50).

CHAPTER 4

DISCUSSION

The anther wall develops prior to meiosis I of the pollen mother cells. Wall development appears to follow the dicotyledonous type, the most common type of wall formation in angiosperms. In it, the tapetum develops directly from secondary parietal tissue while the middle layer and endothecium develop from daughter cells of the primary parietal tissue. This mode of wall formation is found in other members of the Papilionoideae that have been studied (Davis 1966).

The sporogenous tissue appears to undergo only one pre-meiotic mitotic division. The cytoplasm of sporogenous tissue cells has many ribosomes, a few small vacuoles, many mitochondria with poorly developed cristae, electron-dense plastids which show little internal differentiation, apparently active dictyosomes, and fine strands of RER scattered throughout it. In some cells concentric membrane lamellae and "double membrane-bound inclusions" are present. Individual pollen mother cells are connected to each other by plasmodesmata. These observations corroborate those of Echlin and Godwin (1968a) for Helleborus except for an apparent lack of plastids at this stage in Helleborus.

Concentric membrane lamellae and "double membrane-bound inclusions" have been seen only in this time period in Lens and Pisum. Similar structures have been reported in Cephalotaxus spermatozoids just before they penetrate oospheres (Gianordoli 1974). Electron-dense material may also be deposited on the lamellae in Cephalotaxus

but this does not occur in Lens and Pisum. In Gasteria microspores from diplotene of meiosis I to the end of the quartet period, concentric membrane lamellae are present (Willemse 1972). In Lilium studies of the formation of cytoplasmic inclusions during gametogenesis have been made (Dickinson and Andrews 1977; Dickinson and Heslop-Harrison 1977). As pores have been found in these inclusions they are believed to be of nuclear envelope origin (Dickinson and Heslop-Harrison 1977). Pores are also found in the stacked annulate lamellae which are found in some animal cells. Annulate lamellae have also been reported in microsporocytes and mature pollen of Canna (Scheer and Franke 1972). In Lilium "multimembrane bound inclusions" were reported by Dickinson and Andrews (1977) to form around leptotene of meiosis I. Normally only 2 concentric lamellae were found in microsporocytes of Lilium while in megasporocytes, the lamellae disappeared from the cytoplasm during the quartet stage while in microsporocytes, they remained longer.

During interphase before meiosis I in the pollen mother cells of Pisum, large dilatations between the outer and inner nuclear envelopes, are apparent primarily between pores. In Podocarpus evaginations of the outer nuclear envelope, sometimes reaching as far as the plasmalemma, have been seen (Vasil and Aldrich 1970). Small vesicles with dark contents have been found within these evaginations in Podocarpus. These evaginations disappear at the onset of meiosis in Pisum, Lens and Podocarpus. They do not reappear in Lens or Pisum but do in Podocarpus during the quartet stage.

By pachytene of meiosis I in Pisum and Lens, large cytomictic channels have been formed between the meiocytes. These channels have been found in a number of angiosperm genera including Endymion (Angold

1968); Citrus (Horner and Lersten 1971); Helleborus (Echlin and Godwin 1968b); Tradescantia (Waterkeyn 1962); Cannabis and Silene (Heslop-Harrison 1964). Cytomictic channels have not been observed in the gymnosperms Pinus (Dickinson and Bell 1976a; Willemse 1971a) and Podocarpus (Vasil and Aldrich 1970). Perhaps, as Vasil and Aldrich (1970) suggest, lack of cytomictic channels and a reduced amount of callose in the quartet stage may be two features which are characteristic of gymnosperm pollen development. The mode of formation of cytomictic channels has not been studied in any flowering plant. It has been suggested that they do not form at the sites of plasmodesmata (Heslop-Harrison 1964). Cytomictic channels have also been suggested to arise de novo (Heslop-Harrison 1966).

The nucleolar cap which forms during prophase of meiosis I in Pisum and Lens is a distinctive feature of the nucleus at this time. A nucleolar cap has also been reported in Pisum megasporocytes (Wiatr in press); I have also seen it in Lens megasporocytes. In Lilium microspores, a crescentic nucleolus has also been observed (Dickinson and Heslop-Harrison 1970). This crescentic nucleolus may prove to be a feature common to members of the Vicieae tribe of the Papilionoideae during prophase of meiosis I. Its cytological significance cannot be assessed at this time.

Stages in cytokinesis to form the quartets have not been observed in Pisum and Lens; however, I have seen both a binucleate cell and a trinucleate cell during the course of meiosis with no evidence of cell plate formation. From the above observation it would seem to me that cell plate formation is most likely to be of the simultaneous type which is that most commonly found in the Papilionoideae and Leguminosae (Davis 1966).

In Lens only a tetrahedral arrangement of the quartets occurs. In Pisum tetrahedral and decussate arrangements of quartets have been found. Other members of the Papilionoideae usually have tetrahedral, decussate and isobilateral quartets (Davis 1966).

4.1. Callose

4.1.1. Deposition

Stages of development of the callose special wall have not been followed in detail in Lens and Pisum. On the basis of electron microscopical observations and of the light microscopical aniline blue fluorescence method, it would seem that callose is not laid down until late prophase of meiosis I. In Citrus callose is first laid down before prophase of meiosis I (Horner and Lersten 1971). In Cucurbita it is deposited first during leptotene of prophase of meiosis I (Eschrich 1964).

The callose special wall, which purportedly isolates each microspore from other members of the quartet, is a feature found both in angiosperms and gymnosperms. However, in the latter group, the amount of callose deposited seems to be less extensive (Vasil and Aldrich 1970).

4.2.2. Dissolution

There are few references in the literature to the mode of callose dissolution which leads to release of microspores from the quartet into the loculus (cf Eschrich 1964 and 1966).

In Cucurbita as in Lens and Pisum the callose is first dissolved from the corners of the pollen mother cell. In Helleborus callose nearest the tapetum breaks down first (Echlin and Godwin 1968a). The callose special wall becomes infiltrated with numerous channels or

"corrosion canals" in Cucurbita (Eschrich 1964) and in Lens and Pisum. For Cucurbita most callose dissolution is from the outside in (Eschrich 1966). This is what I have found in Lens and Pisum, except on one occasion when dissolution appeared to be bidirectional. Eschrich (1966) has seen pseudopodia-like plasmalemma evaginations extending from the microspore into the callose at the time of callose dissolution. This brief "amoeboid stage" is interpreted by Eschrich (1966) as evidence for the bidirectionality of callose dissolution.

My observation that callose lying closest to the microspore plasmalemma is more electron-dense may be a depositional rather than a degradational phenomenon. In Zea a layered appearance of the callose special wall has been reported (Skvarla and Larson 1966). The appearance of concentric callose layers of different density in Helleborus, Gnetum, Lavatera and Tradescantia has been described at the light microscope level (Waterkeyn 1962 and 1964). Waterkeyn suggests that variations in callose density can be correlated with meiotic stage. In Helleborus and Lavatera callose deposition is centrifugal with alternating pairs of less dense and more dense layers of callose. Three alternating pairs of callose layers form, the first during prophase I, the second during telophase I and the third after cytokinesis. In Gnetum and Tradescantia deposition is the same except that no alternating layer forms during telophase I.

4.2. Tapetum

Two reports in the literature mention tapetal form in either Pisum or Lens. Cooper (1938) says that each tapetal cell in Pisum becomes binucleate. However, in my material I have found that Pisum tapetal cells are uninucleate and only rarely binucleate. Uninucleate

tapetal cells are found in Lens (Shukla 1954). It has been suggested that uninucleate tapetal cells are characteristic of the Mimosoideae and Papilionoideae while multinucleate or binucleate tapeta are characteristic of Caesalpinoideae (Buss 1971). Shukla (1954) states that tapetal cells in Lens exhibit a condition intermediate between secretory and glandular. I have not seen this in Lens. In my specimens, the tapetum remained intact until dissolution at the vacuolate pollen grain stage. At this time, virtually none of the tapetal cytoplasmic contents remain nor are separate organelles usually distinguishable. Secretory tapeta have been reported in all members of Leguminosae examined except for Cassia and Chamaecrista (Buss 1971).

Tapeta are classified as either secretory or periplasmodial. Only one, detailed electron microscopical study has been made of the periplasmodial type in a species of Tradescantia (Mepham and Lane 1969). In Tradescantia radial cell walls begin to break down early in meiosis I. Later in meiosis I, this breakdown is completed and tapetal cytoplasm invades the loculus. In the periplasmodial tapetum of some composites it is not until after callose special wall dissolution that the tapetum invades the loculus (Heslop-Harrison 1969).

Within secretory tapeta the loss of cell walls around tapetal cells occurs at different times: during meiosis I - Avena (Steer 1977); at the end of meiosis - Capsicum (Horner and Rogers 1974); during quartet stage - Citrus (Horner and Lersten 1971), Helleborus (Echlin and Godwin 1968a), Lens, Pinus (Dickinson and Bell 1976b) Pisum, Beta (Hoefert 1971); during early pollen grain development - Silene and Cannabis (Heslop-Harrison 1963b). However in each case tapetal cells remain in situ with their plasmalemmas intact until either just prior

to microspore mitosis in Citrus (Horner and Lersten 1971) and Beta (Hoefert 1971) or after microspore mitosis in all other species described. The tapetum may not finally degenerate until the time of anther dehiscence as in Helleborus (Echlin and Godwin 1968a). At the time of dissolution tapetal cells may be at varying stages of senescence: distinguishable organelles are still present in Capsicum (Horner and Rogers 1974) and Pinus (Dickinson and Bell 1976b); no organelles are distinguishable in Avena (Steer 1977), Helleborus (Echlin and Godwin 1968a), or in Lens and Pisum.

From this discussion, I think it is clear that the distinction between secretory and periplasmodial tapeta needs to be looked at carefully. In both types breakdown of the cell wall may begin about meiosis I. In both dissolution of the plasmalemma and invasion of tapetal contents into the loculus may occur not long after callose special wall dissolution. Perhaps, then, more emphasis should be placed on the state of the tapetum at dissolution. I suggest that periplasmodial tapeta are those which retain nuclei and "normal" cytoplasm at the time of dissolution while secretory tapeta lack nuclei and possess degenerate cytoplasm at dissolution.

During interphase before meiosis I, tapetal cells become quite distinctive in anthers of Pisum and Lens. A tapetal cell is characterized by the following features: prominent interphase nucleus with one, rarely two, nucleoli; small vacuoles around the nucleus; a convoluted plasmalemma; cytomictic channels along radial walls; few lamellae in plastids which have lipid and/or starch inclusions; apparently active dictyosomes; inflated strands of RER and some SER which both ramify throughout the cell. In both Helleborus (Echlin and Godwin 1968a) and

Pinus (Dickinson and Bell 1972 and 1976a) no lipid is found in the plastids at this stage, small vacuoles are not found clustered around the nucleus, the plasmalemma is not convolute, and cytomictic channels do not occur along radial walls of the tapetal cells. Further differences are found in the individual plants - Pinus has two types of vesicles present in the cytoplasm and also exhibits an increase in mitochondrial size and number at this time (Dickinson and Bell 1976a). Helleborus has many microtubules near the cell wall, multi-vesicular bodies outside the plasmalemma and pro-Ubisch bodies which are believed to be of endoplasmic reticulum origin are found in the cytoplasm (Echlin and Godwin 1968a).

At meiosis there seems to be greater dissimilarity between the tapetal cytoplasm of the individual species that have been studied. The cytoplasm of Pisum and Lens, as described above, is distinct from that of the other species studied: Pinus (Dickinson and Bell 1972 and 1976b), Avena (Steer 1977), Helleborus (Echlin and Godwin 1968a) and Podocarpus (Vasil and Aldrich 1970).

At the quartet stage, there is little similarity between the tapetum in Lens and Pisum and that observed for others - Pinus (Dickinson and Bell 1972), Podocarpus (Vasil and Aldrich 1970), Citrus (Horner and Lersten 1971), Capsicum (Horner and Rogers 1974) and Lilium (Heslop-Harrison 1969). There are some correlations with that of Helleborus: highly convoluted nature of tapetal cell wall; breaking down of the ITW; presence of abundant RER and SER of which some of both lies near the ITW. Abundant RER and SER is also found in Beta at this stage (Hoefert 1971). The apparent main difference between Pisum and Lens and other species except Ipomoea (Godwin et al. 1967) at this time is

the occurrence of stages of orbicule development which are absent in my specimens: in Pinus, sporopollenin is being laid down on the pro-orbicules (Dickinson and Bell 1972); in Helleborus the pro-orbicules are initially surrounded by a zone of ribosomes but later these pro-orbicules are released from the tapetum into the loculus and deposition of electron-dense material upon them occurs (Echlin and Godwin 1968a); in Podocarpus sporopollenin droplets aggregate on the plasmalemma (Vasil and Aldrich 1970); in Citrus pro-orbicular bodies lie between the tapetal plasmalemma and the disappearing cell walls (Horner and Lersten 1971); in Capsicum tapetal cell walls are replaced by a layer of orbicules which have the same electron-density as the material being deposited on the exine (Horner and Rogers 1974); in Lilium the pro-orbicules are all extruded from the tapetum before the end of the quartet stage (Heslop-Harrison and Dickinson 1969).

In the early vacuolate pollen grain stage, I have seen electron-dense bodies clustered along the radial walls of tapetal cells. These may be orbicules. In other species studied, two modes of formation of orbicules have been proposed: condensation of electron-dense material around a less electron-dense core (Steer 1977; Dickinson and Bell 1972; Heslop-Harrison and Dickinson 1969; Rowley 1963; Horner and Lersten 1971) or lamellar deposition around a less electron-dense core (Risueno et al. 1969; Willemse 1971a). Neither of these modes of origin would seem to apply in Pisum and Lens. After a light microscope survey of tapeta in Leguminosae, Buss (1971) reported the absence of orbicules in the species he examined.

The role of orbicules is not fully understood. In Lilium it has been suggested that they may provide an hydrophobic surface so that

pollen is easily detached at dehiscence (Heslop-Harrison and Dickinson 1969). In Ficaria (Roland 1967) and Allium (Risueno et al. 1969) orbicules are reported to occur in the exine. This would suggest a role in exine formation; however the presence of orbicules in the exine has not been reported in other species. The variability of observations suggest that there is not a common mode of sporopollenin deposition. In genera where direct transfer is not observed, perhaps, as Heslop-Harrison (1963a) has postulated, the orbicules are either temporary storage sites for sporopollenin or the by-products of sporopollenin synthesis which will not be re-mobilized.

In the course of senescence in Lens and Pisum, tapetal cells are initially nucleated and nucleolated; have a large vacuole; possess lipid-containing plastids both with and without starch; contain ramifying hair-like strands of RER throughout. Next, lipid droplets accumulate along the radial wall, small vacuoles develop and the RER proliferates. The stacked arrangement of the RER that occurs in Pisum and Lens has also been reported in Paeonia (Marquardt et al. 1968). However I have not observed an association of plastids and ER as is found in Paeonia. Next, in Lens and Pisum, the nucleus starts to break down and the cytoplasm becomes more vacuolate; plastids no longer contain starch and some of them begin to lyse; lipid droplets are found around the circumference of degenerating mitochondria; much of the RER has disappeared; the ITW is convoluted with most of the plasmalemma intact; electron-dense lipid droplets and "spiny" vesicles are found near the plasmalemma; small fragments of cytoplasm break off into the loculus. This release of small fragments of cytoplasm into the loculus prior to plasmalemma dissolution has also been observed in Pinus (Dickinson and

Bell 1976b). Once the nucleus disappears in Pisum and Lens, the cytoplasm becomes more diffuse; most mitochondrial contents disappear but electron-dense lipid droplets still occur along the periphery; RER comes to lie along the ITW and OTW; the plasmalemma remains intact. Final dissolution of tapetal remnants into the loculus does not occur until the vacuolate pollen grain stage.

4.3. Angiosperm Primexine Development

Primexine development in Pisum and Lens shows similarities to that described for several other species including: Lilium (Heslop-Harrison 1968c and Dickinson 1970); Saponaria and Silene (Heslop-Harrison 1963a); Zea (Skvarla and Larson 1966); Linum (Vazart 1970); Ipomoea (Godwin et al. 1967); Cannabis and Melandrium (Heslop-Harrison 1963b); Helianthus (Horner 1977).

In Linum a "parietal layer" believed to be of vacuolar origin is formed outside the microspore plasmalemma before the fibrillar primexine matrix (Vazart 1970). I have found no evidence of this in either species I have studied nor otherwise in the literature at this developmental stage. Eschrich (1964) described a similar method of wall formation prior to callose special wall development in Cucurbita.

In Zea before fibrillar primexine matrix development, the plasmalemma interaperturally withdraws from the callose special wall. Endoplasmic reticulum is found in prospective aperture areas. There are tubular evaginations of the plasmalemma into the space between the callose special wall and microspore surface. Endoplasmic reticulum is believed to be involved in plasmalemma tubule formation. Many active-appearing dictyosomes are also seen in the area.

The primexine matrix is laid down interaperturally in Pisum and Lens. The same has been observed for Saponaria and Silene (Heslop-Harrison 1963a); Cannabis and Melandrium (Heslop-Harrison 1963b); Lilium (Heslop-Harrison 1968c); Helleborus (Echlin and Godwin 1969); Helianthus (Horner 1977); Sorghum (Christensen et al. 1972); Beta (Hoefert 1969a); Parkinsonia (Larson and Lewis 1962). This corresponds to the "Silene type" of aperture formation (Rowley 1975). No cytoplasmic associations in aperture formation were noted in Pisum and Lens. This is in contrast to the situation in Helianthus, where primexine development is thought to be prevented in aperture regions by tubules of endoplasmic reticulum origin (Horner 1977). A similar observation was made for Silene and Saponaria, where plates of endoplasmic reticulum come to lie near the plasmalemma in the aperture area (Heslop-Harrison 1963a). In Lilium fragments of endoplasmic reticulum are involved in aperture formation (Dickinson 1970).

Although I have seen an undulate plasmalemma, neither tubules nor packets of vesicles as found in Lilium (Dickinson 1970) have been observed in the fibrillar primexine matrix. I do however agree with Dickinson's (1970) and Vazart's (1970) reports that there is no association of cytoplasmic organelles with probaculum formation. Linum (Vazart 1970) and Ipomoea (Godwin et al. 1967) have gaps in the primexine matrix where probaculae will form. I have not observed these in Pisum and Lens.

In Silene, Melandrium, Cannabis and Saponaria endoplasmic reticulum is associated with developing probaculae (Heslop-Harrison 1963b). I have seen one such an association in Pisum. Vazart (1970) reported an association between the developing probaculae and mitochon-

dria in Linum. Godwin et al. (1967) believed that vesicles were involved. In Lens I have seen vesicles lying between the primexine and plasmalemma during probaculum formation. Plasmalemma evaginations also penetrated into the base of some probaculae in this species. Dickinson (1970) has seen similar structures in Lilium; in LS they have a lamellate form, in TS a myelin arrangement of sheets. As in Lilium (Dickinson 1970) and Helleborus (Echlin and Godwin 1969), initial probaculae formation takes place in Lens and Pisum along microspore contact points.

4.4. Angiosperm Exine Development

At the time of callose dissolution, and release of Lens and Pisum microspores from the quartet, sporopollenin deposition begins. This deposition first occurs on the outer primexine surface as in Silene, Melandrium, Saponaria, Cannabis (Heslop-Harrison 1963b). Probaculae become more electron-dense and tectum formation begins. Next the nexine 1 layer, which lies closest to the plasmalemma begins to develop. In the early stages of nexine development scattered groups of baculae become joined at their bases by electron-dense material. In Endymion nexine 1 formation is believed to occur via deposition of sporopollenin lamellae (Angold 1968). This does not seem to happen in Lilium (Dickinson 1970) nor in Pisum or Lens.

I have not followed details of nexine 2 formation. However, just prior to intine formation, I have twice seen either vesicles with electron-dense peripheral deposits or electron-dense deposits alone between the plasmalemma and nexine 2 layers. These may have been involved in either nexine 2 or intine formation. In Populus and Salix vesicles seem to be involved in nexine 2 and intine formation (Rowley

and Erdtman 1967). In Helleborus dictyosomes are also thought to be involved in intine formation (Echlin and Godwin 1969). In contrast a lamellar method of formation is described for nexine 2 in Ipomoea (Godwin et al. 1967); Lilium (Dickinson and Heslop-Harrison 1968); Anthurium (Rowley and Southworth 1967); Silene, Melandrium, Saponaria and Cannabis (Heslop-Harrison 1963b).

I have not seen exine interbedding in the narrow fibrillar intine as described in Helleborus (Echlin and Godwin 1969). Nor have I seen channels in the nexine 1 and tectum like those reported in Zea (Skvarla and Larson 1966). As in Helleborus (Echlin and Godwin 1969) and Zea (Skvarla and Larson 1966), a decrease in electron-density occurs with age in Pisum and Lens.

4.5. Gymnosperm Exine Development

Although some observations have been made on the fine structure of mature gymnosperm pollen exines (Gullvåg 1966; Lepousé 1969), there have been few developmental studies. It has been suggested that terminology related to angiosperm exine development should not be applied to gymnosperm exine development (Gullvåg 1966).

In Podocarpus the primexine matrix is made up of 2 layers, an outer fibrillar one with a loose reticulate network and an inner one in which the fibrils lie parallel to the plasmalemma (Vasil and Aldrich 1970). In Pinus (Willemse 1971b) as in Lens and Pisum only a single fibrillar layer forms. In Podocarpus (Vasil and Aldrich 1970), Pinus (Willemse 1971b) and Ginkgo (Rohr 1977) the tectum begins to develop first. This is in contrast to Pisum and Lens where probaculae form first. In Ginkgo (Rohr 1977), Pinus (Willemse 1971b) and Podocarpus (Vasil and Aldrich 1970) the "baculae" begin to grow in towards the

microspore plasmalemma from the tectum. Lamellar deposition of the nexine 1 occurs next in Taxus and Ginkgo (Rohr 1977), Pinus (Willemse 1971b) and Podocarpus (Vasil and Aldrich 1970). Nexine 2 and 3 layers form by coalescence of small granules in Podocarpus (Vasil and Aldrich 1970) while nexine 2 forms by accumulation of electron-dense lamellae in Ginkgo (Rohr 1977). In angiosperms a lamellar mode of nexine 1 formation has been found in Endymion (Angold 1968) and of the nexine 2 in Ipomoea (Godwin et al. 1967), Lilium (Dickinson and Heslop-Harrison 1968), Anthurium (Rowley and Southworth 1967), Silene, Melandrium, Saponaria and Cannabis (Heslop-Harrison 1963b).

In Podocarpus (Vasil and Aldrich 1970) the narrow intine is not fibrillar; however, Lens and Pisum intines have a fibrillar nature. Dictyosome-derived vesicles are thought to be involved in intine formation in Podocarpus (Vasil and Aldrich 1970). In the angiosperms, Populus, Salix (Rowley and Erdtman 1967) and Helleborus (Echlin and Godwin 1969) dictyosome vesicles are also apparently involved in intine formation.

4.6. Mature Pollen Cytoplasm

4.6.1. Vegetative cell

Studies of mature pollen cytoplasm have been made in only a few species: Epidendrum (Cocucci and Jensen 1969); Gossypium (Jensen et al. 1968, Fisher et al. 1968); Haemanthus (Sanger and Jackson 1971b); Petunia (Kroh 1967, Sassen 1964); Hippeastrum, Hymenocallis, Parkinsonia and Ranunculus (Larson 1965); Impatiens (van Went 1974); Lycopersicum (Cresti et al. 1975).

Like that of Gossypium, the cytoplasm of Lens can be divided into 2 regions: outer and inner. Cytoplasmic zonation is also seen in Pisum, but is less well-defined. The outer zone of cytoplasm in Lens and

Gossypium is similar. There are many mitochondria, dictyosomes and, vesicles but no lipid droplets. The main difference is in the arrangement of the endoplasmic reticulum which forms a pocket in Gossypium and a bilayer in Lens. Pisum cytoplasm resembles that of Lens except for a lack of vesicles and the presence of some lipid droplets.

The inner or core cytoplasm of all species studied has dictyosomes, mitochondria and plastids with simple lamellae. Vesicles are not a distinctive feature of the inner cytoplasm in Pisum and Lens. The RER consists of fine strands in Pisum. In Lens the parallel cisternae of ER have an electron-dense, probably lipid layer between them with ribosomes studding the outer ER surface. The ER cisternae are most commonly associated with vacuoles or electron-dense bodies. A similar arrangement of RER is reported for Impatiens (van Went 1974); Lycopersicum (Cresti et al. 1975); Castilleia, Cordylanthus, Ophiocephalus and Orthocarpus (Jensen et al. 1974). In none of these genera is RER associated with vacuoles as it is in Lens. RER is associated with both electron-dense bodies and plastids in Castilleia, Cordylanthus, Ophiocephalus and Orthocarpus (Jensen et al. 1974). A stacked arrangement of RER occurs in Lycopersicum (Cresti et al. 1975); Castilleia, Cordylanthus, Ophiocephalus and Orthocarpus but not in Lens. In Gossypium the RER forms a pocket rather than a bilayer. The inner contents of the pocket are lipid droplets and vesicles which contain protein, lipid and carbohydrate (Fisher et al. 1968).

The azonal cytoplasm in Epidendrum, Hippeastrum, Hymenocallis, Parkinsonia and Ranunculus is otherwise very similar to that in Pisum and Lens. The plastids are not seen associated with the ER in the species I have studied. In Pisum vesicles are not usually closely associated with dictyosomes.

In Petunia (Kroh 1967, Sassen 1964) and Impatiens (van Went 1974) the plastids have well-developed starch grains while those in Pisum and Lens do not. Mitochondria are not usually evenly distributed and some vacuoles are present in the cytoplasm of Pisum and Lens.

Haemanthus cytoplasm differs from that of Pisum and Lens in that it lacks lipid bodies, possesses vesicular ER, and vesicles with lipid inclusions, and polyribosomes (Sanger and Jackson 1971b).

4.6.2. Generative cell

The generative cell cytoplasm in Pisum and Lens is similar to that of other species studied: Beta (Hoefert 1969b), Dactylorchis (Heslop-Harrison 1968b), Epidendrum (Cocucci and Jensen 1969), Gossypium (Jensen et al. 1968), Haemanthus (Sanger and Jackson 1971a), Hippeastrum (Larson 1965), Petunia (Sassen 1964) and Secale (Karas and Cass 1976).

I have not observed microspore mitosis and the early stages of development in Pisum and Lens generative cells. In Epidendrum (Cocucci and Jensen 1969), Petunia (Sassen 1964), Hordeum (Cass and Karas 1975) and Secale (Karas and Cass 1976) a cell wall has been reported to be present around the generative cell. In Pisum and Lens this was also observed. No plasmodesmata have been seen between the generative and vegetative cells in Pisum and Lens. They are, however, reported for Epidendrum (Cocucci and Jensen 1969), Dactylorchis (Heslop-Harrison 1968b) and Secale (Karas and Cass 1976). The ER in Pisum and Lens occurs in fine hair-like strands unlike that of Parkinsonia (Larson 1965) and Epidendrum (Cocucci and Jensen 1969) which is dilated. In Petunia the ER is connected to the nuclear envelope (Sassen 1964); such a relationship has not been found in Pisum and Lens. Nor is ER arranged parallel to the plasmalemma in Pisum and Lens as it is in Gossypium

(Jensen et al. 1968) and Impatiens (van Went 1974). No dictyosomes were seen in Dactylorchis (Heslop-Harrison 1968b); however, they have been reported in all other species studied including in this investigation.

No recognisable plastids have been seen in the generative cytoplasm of Pisum or Lens. This lack of plastids in the generative cytoplasm has been confirmed in the majority of species studied: Dactylorchis (Heslop-Harrison 1968b), Epidendrum (Cocucci and Jensen 1969), Gossypium (Jensen et al. 1968), Haemanthus (Sanger and Jackson 1971a), Hordeum (Cass and Karas 1975) Impatiens (van Went 1974), Lycopersicum (Cresti et al. 1975), Mirabilis (Lombardo and Gerola 1968), Parkinsonia (Larson 1965), Petunia (Sassen 1964) and Secale (Karas and Cass 1976).

There are, however, a few reports of the presence of plastids in the generative cell: Beta (Hoefert 1969a), Hippeastrum (Larson 1965), Lobelia (Dexheimer 1965), Pelargonium (Lombardo and Gerola 1968) and Solanum (Clauhs and Grun 1977).

Why plastids are not normally passed onto the zygote by both male and female gametophytes is problematic. At this stage we have no satisfactory explanation for the phenomenon. In Solanum (Clauhs and Grun 1977) it has been postulated that some "lethality factor" is in operation. This factor is supposed to explain the loss of plastids that were present in the young generative cell. Why this "lethality factor" is specific for only generative cell plastids in pollen is not known.

4.7. Endothecium

Fibrous thickenings frequently occur in mature endothecial cells but little else is known about them. De Fossard (1969) made a light

microscope study of endothecial cell thickenings in Chenopodium rubrum; this is apparently the most recent work since that of Kuhn (1908). Kuhn described endothecial thickenings in representatives of 180 angiosperm families. In 45.5% of the genera studied "manubria type" thickenings occur. This type is characterized by presence of partly to completely thickened ITWs and rods extending from the ITW to the OTW. His study included 18 genera from the Leguminosae, including Lathyrus from the Vicieae. All genera that he examined had well-developed thickenings. The inner tangential wall is variously thickened: in Coronilla very little so that stellate figures are observed; in Barcleya and Glycine it is thickened more; in Acacia and Lathyrus it is entirely thickened. In Pisum and Lens I have found that the ITW is entirely thickened as in Lathyrus.

De Fossard (1969) noted the rapid development of endothecial cell thickenings and the difficulty of securing intermediate stages. I have found that the endothecium develops thickenings over a short period of time in Pisum and Lens. When the endothecial thickenings first appear early in pollen development there seems to be little fibrillar material present. Just prior to dehiscence much fibrillar material is evident within the thickenings. At this time endothecial cell cytoplasm is senescing. Mepham and Lane (1970) have suggested that endothecial cell thickenings are secreted by a smooth membrane complex.

The composition of the endothecial cell thickenings is uncertain. According to Vasil (1967), the endothecial cells "are radially elongated and fibrous bands of callose" arise from the ITW. In Lens and Pisum the endothecial cells showed no reaction to the aniline blue fluores-

cence method for callose. The appearance of numerous fibrils in mature thickenings also suggests that they are not primarily callosic since callose is amorphous.

The thickenings do not react to Sudan black b which is used to indicate the presence of lipids. The mechanism of action of this test and its degree of lipid specificity are not fully known (Fredericks 1977). The thickenings also do not show an increase in electron-density with osmium post-fixation. It would seem that they do not contain significant amounts of lipid.

Both Lens and Pisum endothecial cell thickenings show a faint reaction to ruthenium red. This test is not entirely specific for pectins as it relies on a particular functional group arrangement that may be present in other compounds (Sterling 1970). In Lens a faint positive reaction is obtained with the hydroxylamine ferric chloride procedure for esterified pectins (Reeve 1959); there is no reaction in Pisum. This result tentatively suggests that Lens endothecial thickenings do contain small amounts of pectin.

A faint reaction occurred in thickenings of both species with the zinc-chlor-iodide procedure (Rawlins and Takashi 1952). The mechanism of this procedure of testing for cellulose is not fully understood. According to Jensen (1962), after acid hydrolysis the iodine accumulates within the cellulose molecule. In the IKI-H_2SO_4 test (Jensen 1962), I had a positive reaction for lignin but not cellulose. The mechanism of this test is believed to be the same as that already outlined for the zinc-chlor-iodide procedure. Given that hemicellulose may behave in the same way as cellulose and that chitin, suberin and lignin may all block the reaction (Jensen 1962), the

reliability of the IKI-H_2SO_4 test is in question. The thickenings are bright under the polarizing microscope. This test relies on the specific orientation of cellulose microfibrils. On the basis of the results of these tests, it seems that some cellulose occurs in endothecial thickenings of Pisum and Lens.

In the Maule (De Fossard 1969) and chlorine-sulphite (cf Appendix II) reactions, if a significant amount of syringyl propane units are present, a positive reaction takes place. Neither reaction mechanism is known (Ludwig and Sarkanen 1971). Syringyl propane units may not be present in weakly lignified tissue (De Fossard 1969). Hence a negative reaction, such as I have seen in the results of the Maule and chlorine-sulphite reactions may not necessarily indicate the absence of lignin. Schiff's reagent (McLean and Cook 1941) reacts with the aldehyde grouping of lignin without periodic acid oxidation. This reagent is specific for the aldehyde functional group; if a positive reaction occurs it does not specifically denote lignin presence (Jensen 1962). Hence the negative reaction would seem to indicate that lignin is absent. Phloroglucinol in HCl (Jensen 1962) reacts with the coniferaldehyde groups and is universally applicable to all lignins. Its action may be masked by a high concentration of syringyl propane units (Ludwig and Sarkanen 1971). Lack of reaction with the Maule and chlorine-sulphite tests would seem to discount the possibility of syringyl propane units masking the phloroglucinol/HCl reaction. Hence it would seem on the basis of this reaction that lignin is not present in Lens and Pisum. Fluorescence of lignin under UV light relies on the presence of double bonds in the lignin polymer. According to Ludwig and Sarkanen (1971) fluorescence under UV light is the most accurate

determination for lignin. Lens does fluoresce under these conditions but Pisum does not. These results suggest that Lens contains some lignin in its endothecial thickenings.

The endothecium, which usually contains well-developed thickenings has been implicated in anther dehiscence. In some cleistogamous flowers endothecial thickenings are absent (Schmid 1977); however, in Pisum and Lens endothecial thickenings are well-developed. Perhaps cleistogamy is a recent adaption of these genera from an outbreeding habit.

4.8. Conclusions

The results obtained in this investigation agree with previous work on members of the Leguminosae. Anther and pollen development within the family as a whole seems to be relatively homogeneous. The majority of family members studied have secretory tapeta, dicotyledonous anther wall development, binucleate pollen at dehiscence, simultaneous cytokinesis to form quartets and well-developed endothecial cell thickenings.

There are however still many questions that remain unanswered for example - how do the endothecial cell thickenings develop?; how are cytomictic channels formed?; why are plastids usually absent from the generative cell?; what is the involvement, if any, of orbicules in sporopollenin deposition? Perhaps future workers may solve these intriguing problems.

Abbreviations used in Figure captions

A	aperture
C	crystal
CC	cytomictic channel
CW	cell wall
D	dictyosome
DQ	decussate quartet
E	epidermis
En	endothecium
FL	fibrillar layer
G	generative nucleus
M	mitochondrion
ML	middle layer
Mn	micronucleolus
N	nucleus
N1	nexine 1
N2	nexine 2
Nu	nucleolus
P	plastid
Pb	probaculum
Pd	plasmodesm
PMC	pollen mother cell
PP	primary parietal layer
RER	rough endoplasmic reticulum
RW	radial cell wall
SP	secondary parietal tissue
SV	"spiny" vesicle
T	tapetum
Te	tectum
TMR	tapetal and middle layer remains
TQ	tetrahedral quartet
V	vesicle
Va	vacuole

Fig. 1: Light micrograph of Pisum showing 2 daughter cells of primary parietal cell division. These form secondary parietal tissue. Phase contrast. x2000.

Fig. 2: Light micrograph of Pisum loculus with pre-meiotic interphase pollen mother cells. The tapetum no longer morphologically resembles the PMC's. Note vacuoles around tapetal nucleus. Phase contrast. x800.

Fig. 3: Light micrograph of Pisum anther with 3-4 layered wall. Anaphase of mitosis of a secondary parietal cell (arrow) to form externally the endothecium and internally the middle layer illustrated. At this stage the tapetum morphologically resembles the pollen mother cells. Phase contrast. x800.

Fig. 4: Light micrograph of Pisum anther with 2-layered wall. Outer epidermal layer and inner primary parietal. Phase contrast. x800.

Fig. 5: Light micrograph of Lens anther with 3-layered wall. Anaphase of mitosis to produce secondary sporogenous cell is seen in one cell. The other cells are at pre-mitotic interphase. Phase contrast. x720.

1

Fig. 6: Electron micrograph of Pisum pre-mitotic interphase sporogenous cell. The nucleus has a nucleolus and micronucleolus. Mitochondria have poorly developed cristae and plastids show little internal differentiation. The cells are connected by plasmodesmata. x5950.

Fig. 7: Electron micrograph of Pisum pollen mother cell at pre-meiotic interphase. Large dilatations (arrow) appear to occur between outer and inner nuclear envelopes, primarily between pores. Dilated rough endoplasmic reticulum cisternae occur in the cytoplasm. x3900.

Fig. 8: Electron micrograph of Pisum 4-layered anther wall consisting of epidermis, endothecium, middle layer and tapetum. Plastids contain starch. Lipid deposits (arrow) occur in epidermis. x2650.

Fig. 9: Electron micrograph of Pisum tapetal cell. It has a convoluted plasmalemma, plastids with few lamellae, apparently active dictyosomes and vacuoles surrounding the nucleus. x5400.

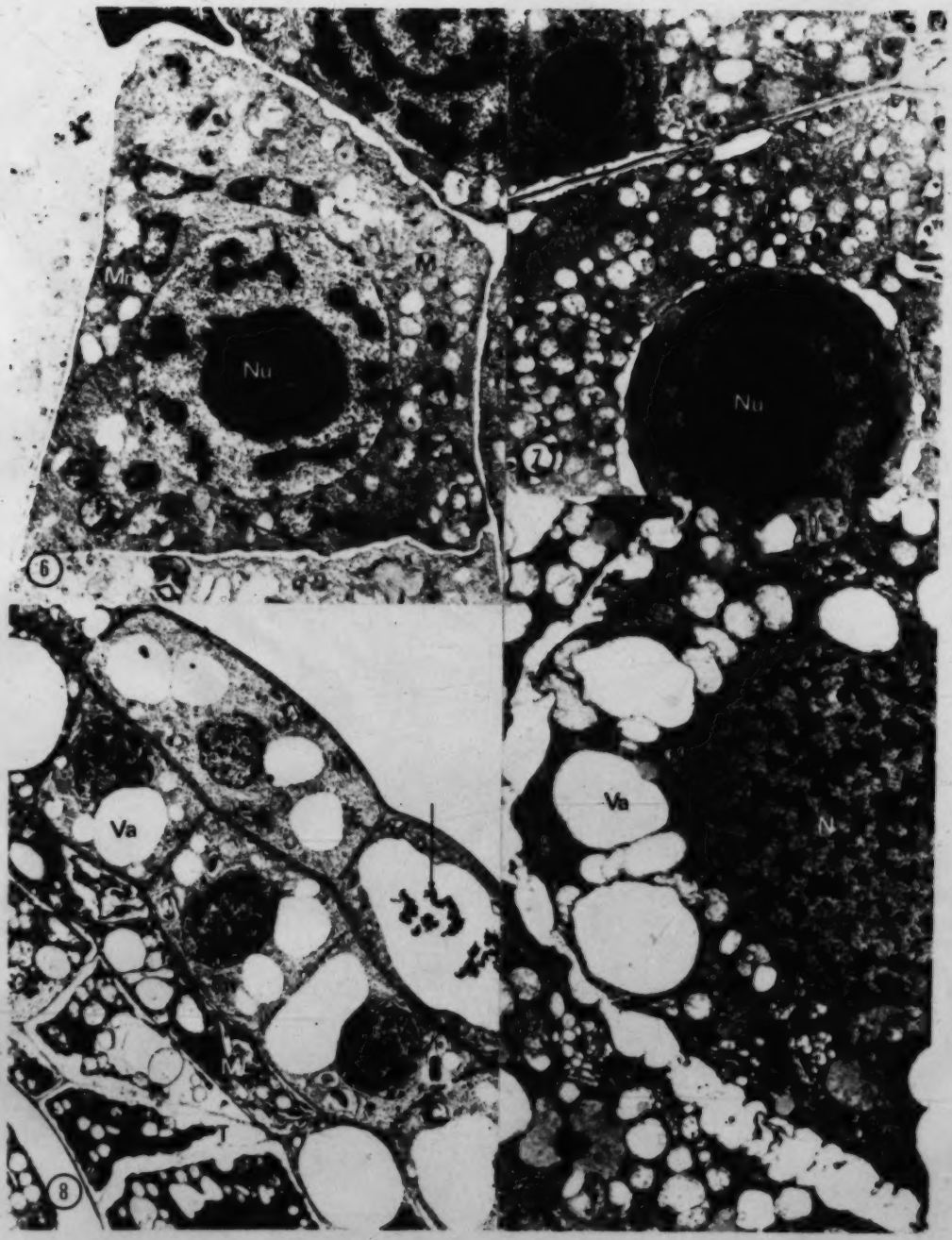

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Fig. 10: Electron micrograph of Lens pollen mother cell at prophase of meiosis I. Note the crescentic nucleolar cap. Crystals of unknown composition and starch containing plastids are seen in the tapetal cells. x3000.

Fig. 11: Light micrograph of Lens pollen mother cell at pachytene of meiosis I. Kidney-shaped nucleolus has darker, outer pars granulosa and lighter inner pars amorpha. Cytomictic channels (arrow) occur between the meiocytes. x1200.

Fig. 12: Light micrograph of Lens anther loculus in which a pollen mother cell is at anaphase of meiosis I. Phase contrast x400.

Fig. 13: Electron micrograph of Pisum pollen mother cell at diplotene of prophase I. An electron-dense crescentic nucleolus and two micronucleoli are present in the nucleus. Cytomictic channels connect the meiocytes. Remnant of severed plasmodesm shown by arrow. Note absence of recognizable middle lamella within the unusual primary wall formation. x3000.

Fig. 14: Electron micrograph showing unusual primary wall formation occurring between meiocytes of Pisum. The middle lamella has broken down. The cell wall is electron-dense while the fibrillar layer is less electron-dense. x7950.

Fig. 15: Electron micrograph of Lens cytomictic channel which has formed between the meiocytes by pachytene. x11400.

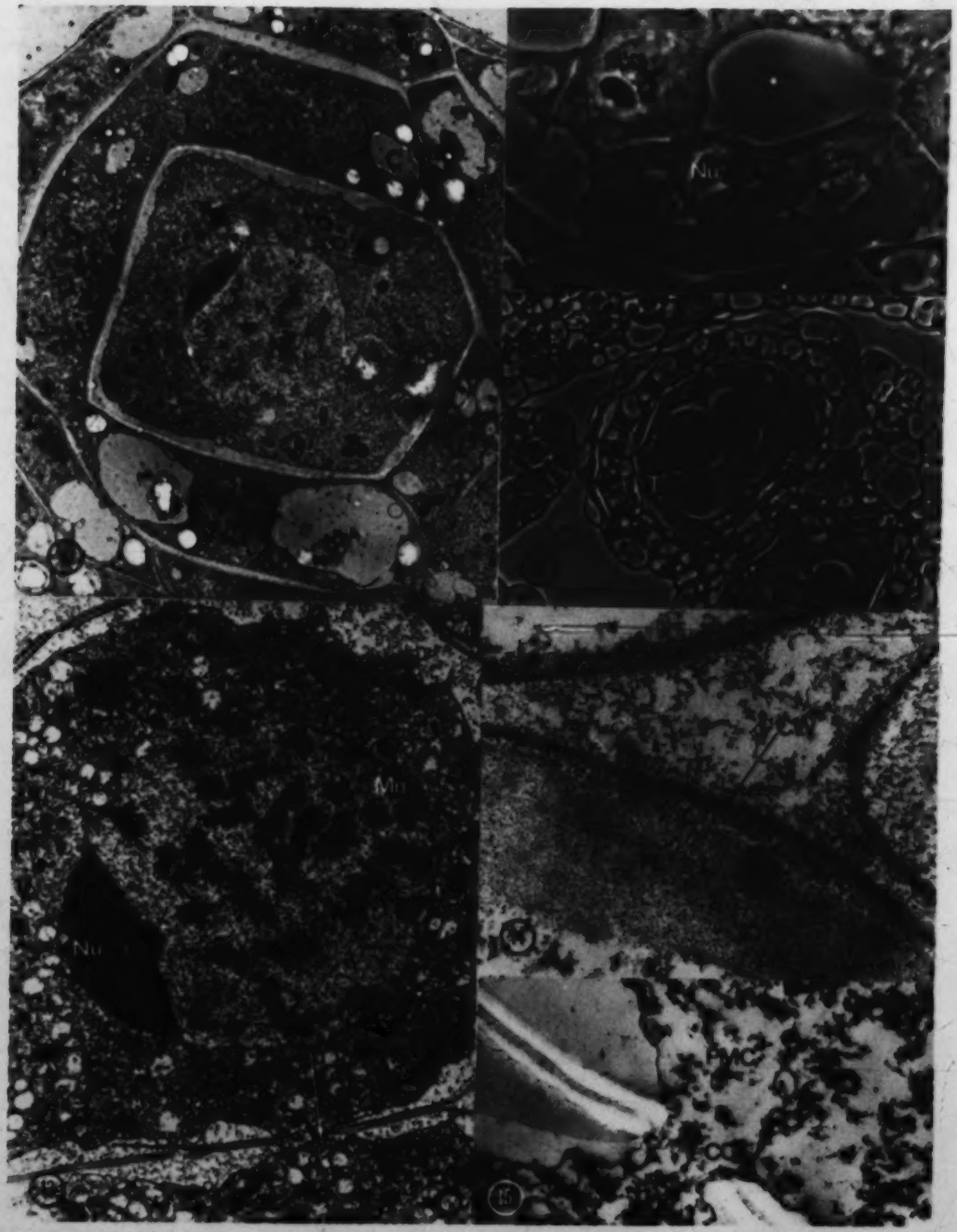

Nu
Mi
Nu
Mi
Nu

Fig. 16: Light micrograph of Lens anther loculus after Sudan black b staining. The epidermal vacuolar inclusions are stained by Sudan black b (arrow). Note lipid droplets within microspore cytoplasm. Phase contrast. x500.

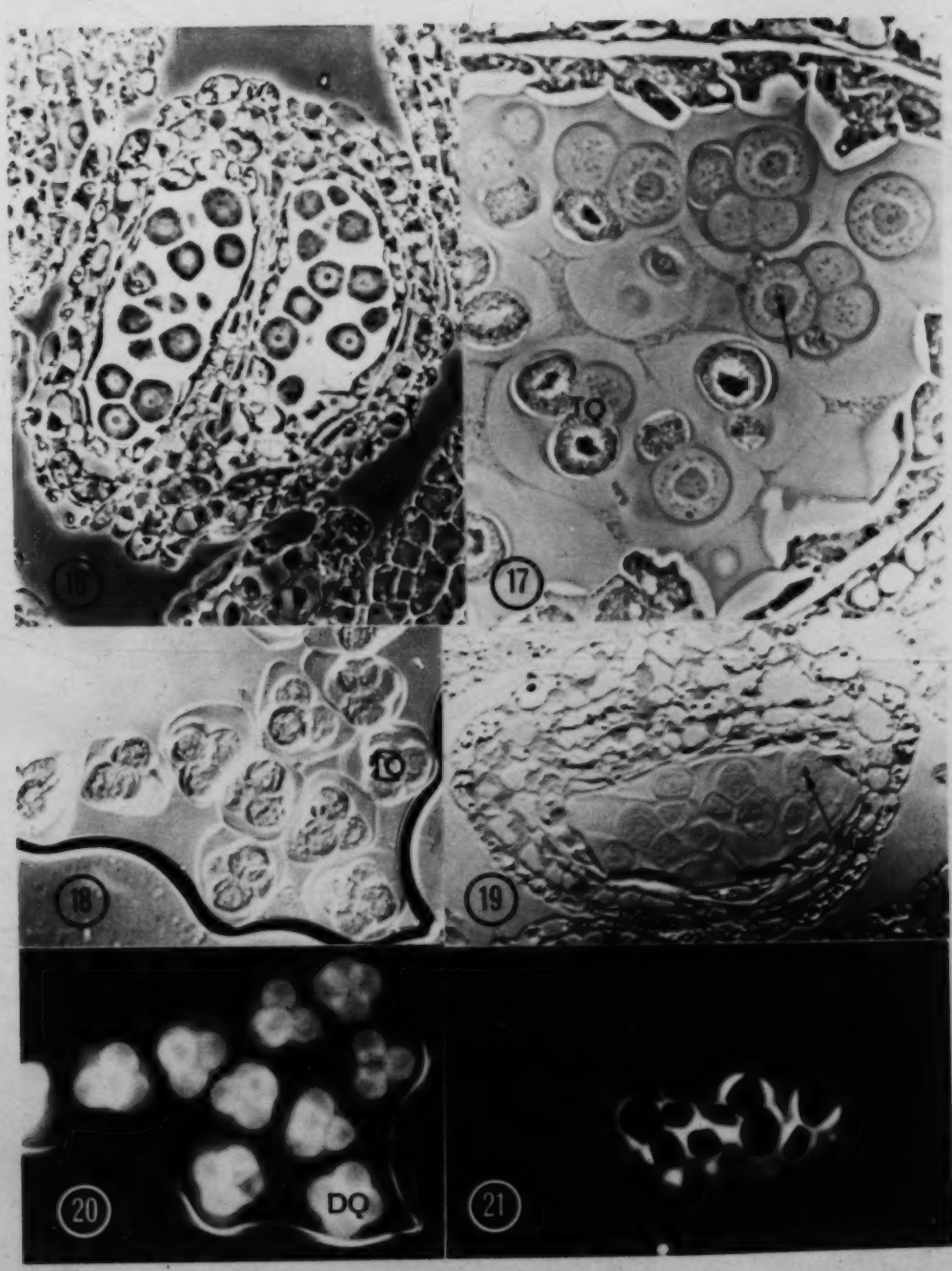
Fig. 17: Light micrograph of Pisum quartets in which the microspores are tetrahedrally arranged. A single nucleolus (arrow) is seen in 2 of the nuclei. Phase contrast. x750.

Fig. 18: Light micrograph of Pisum quartets. Fresh squash preparation. Nomarski-interference. x320.

Fig. 19: Light micrograph of Lens quartets in anther loculus. Note the prominent nucleolus in one of the microspores. Nomarski-interference. x500.

Fig. 20: Fluorescence micrograph of Pisum quartets illustrated in Fig. 18. They are surrounded by a callose special wall. The quartets are tetrahedrally or decussately arranged. x320.

Fig. 21: Fluorescence micrograph of Lens anther loculus illustrated in Fig. 19. The callose pads around the microspores are evident. Callose dissolution is centripetal. x500.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TO
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DQ
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Fig. 22: Electron micrograph of Pisum microspore just prior to complete centripetal callose dissolution. Vacuoles occupy inner cytoplasm around the nucleus. Exine development is progressing and the tectum is being laid down (arrow). Two apertures are also shown. x13140.

Fig. 23: Electron micrograph of Pisum aperture which is made of fibrillar material and partly covered by fragments of exine (arrow). x7250.

Fig. 24: Electron micrograph of Lens microspore showing centripetal dissolution of the callose special wall. Channels extend into the callose. Nearest the microspores the callose is more electron-dense. Extensions of the plasmalemma (arrow) can be seen in the early stages of probaculum formation. x8000.

Fig. 25: Electron micrograph of Pisum tapetum just prior to dissolution of the callose special wall. The plasmalemma is convoluted. Plastids, vacuoles and rough endoplasmic reticulum, which ramifies throughout the cell are distinctive. x4725.

Fig. 26: Electron micrograph of Lens epidermal cell which possesses electron-dense vacuolar inclusions. A larger inclusion occurs in the central portion of the vacuole and smaller ones peripherally (arrow). x3500.

Fig. 27: Electron micrograph of Pisum tapetum in which the radial cell wall has started to break down. A vacuole with cytoplasmic inclusions lies near each nucleus; apparently active dictyosomes are present. x4500.

22
Va
24
Va
Va

Fig. 28: Light micrograph of Pisum microspores just prior to dissolution of the callose special wall (arrow). The microspores are circular in transverse section and elongate in longitudinal section. The tapetal cells now extend well into the loculus. Phase contrast. x400.

Fig. 29: Electron micrograph of Pisum in the early stages of primexine formation. A fibrillar layer forms over the microspore surface except where the aperture will form. Probaculae (arrow) radiate from the microspore plasmalemma. x24300.

Fig. 30: Electron micrograph of Pisum electron-dense probaculae at the base of which initial stages of nexine 1 formation are discernible (arrow). The tectum is also being laid down. x19000.

Fig. 31: Electron micrograph of Pisum probaculae. They have enlarged and are almost wedge-shaped. x16000.

L
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En
28
Pg
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31

Fig. 32: Electron micrograph of Pisum microspore just prior to mitosis. Formation of the nexine 1 layer (arrow) is complete and thickest near the aperture. x3740.

Fig. 33: Electron micrograph of Pisum tapetal cell. It has a prominent interphase nucleus, large vacuole, lipid containing plastids both with and without starch grains (arrow) and fine strands of rough endoplasmic reticulum ramify throughout. x8070.

Fig. 34: Electron micrograph of Lens endothecium which has bulbous wall thickenings (arrow). The plastids lack starch. x7960.

Fig. 35: Electron micrograph of Pisum tapetum. Electron-dense lipid droplets are found along the radial walls (arrow). Rough endoplasmic reticulum is well-developed and small vacuoles are present. x5270.

(32)

Fig. 36: Electron micrograph of Lens tapetum after disappearance of the nucleus. The cytoplasm is diffuse and electron-dense lipid droplets surround the mitochondria. Rough endoplasmic reticulum strands are aligned along the outer and inner tangential walls. x9000.

Fig. 37: Electron micrograph of Pisum tapetum. The cytoplasm is very diffuse and vacuoles are present. Lipid droplets surround degenerating mitochondria. The plasmalemma is still intact. x7900.

Fig. 38: Electron micrograph of Lens tapetum in which the plasmalemma is still largely intact (arrow) despite disappearance of the nucleus and the small amount of cytoplasm remaining. x5850.

Fig. 39: Electron micrograph of Pisum tapetum inner tangential wall. Electron-dense lipid droplets (arrow) and "spiny" vesicles are found near the convoluted plasmalemma. x17520.

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N2
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V
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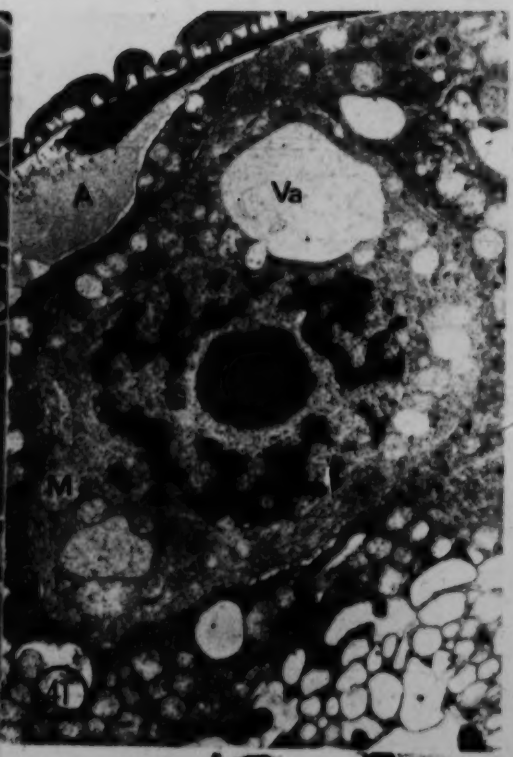
Fig. 40: Light micrograph of Pisum vacuolate pollen grains. The electron-dense vegetative nucleolus (arrow) and interphase generative nucleus can be seen. Tapetal and middle layer remnants have been sloughed off into the loculus. Phase contrast. x690.

Fig. 41: Electron micrograph of Pisum generative cell which has a prominent interphase nucleus. A vacuole and mitochondria are present in the cytoplasm. x7420.

Fig. 42: Electron micrograph of Lens in which transfer of tapetal and middle layer remains is occurring on the pollen grain tectum and interbacularly. x24570.

Fig. 43: Electron micrograph of electron-dense Pisum tapetal and middle layer remains in situ. x3500.



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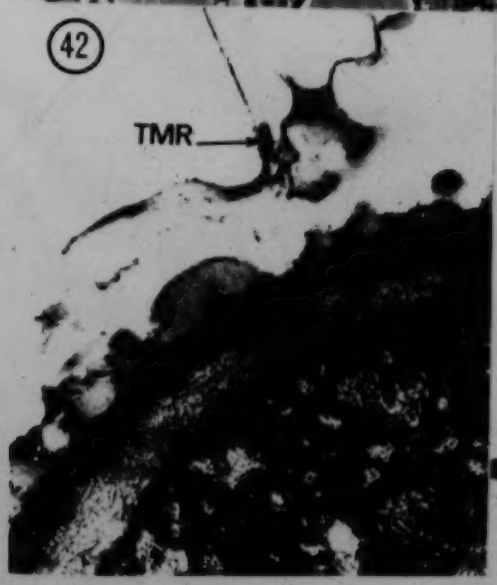

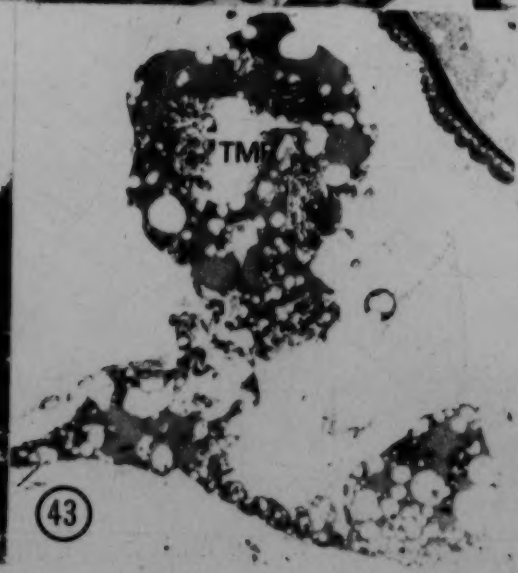

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Fig. 44: Light micrograph of Lens anther loculus with mature pollen grains and rod-shaped endothecial thickenings (arrow). The tapetum and middle layer have completely disappeared. Phase contrast. x640.

Fig. 45: Electron micrograph of Pisum exine prior to intine formation. Vesicles with electron-dense peripheral deposits or electron-dense deposits alone lie between the plasmalemma and nexine 2 layer. x1900.

Fig. 46: Light micrograph of Lens mature pollen after Sudan black b staining. Interbacular lipid deposition has occurred (arrow). The core cytoplasm has numerous lipid droplets. Phase contrast. x800.

Fig. 47: Electron micrograph of Lens fibrous endothecial thickening. x17600.

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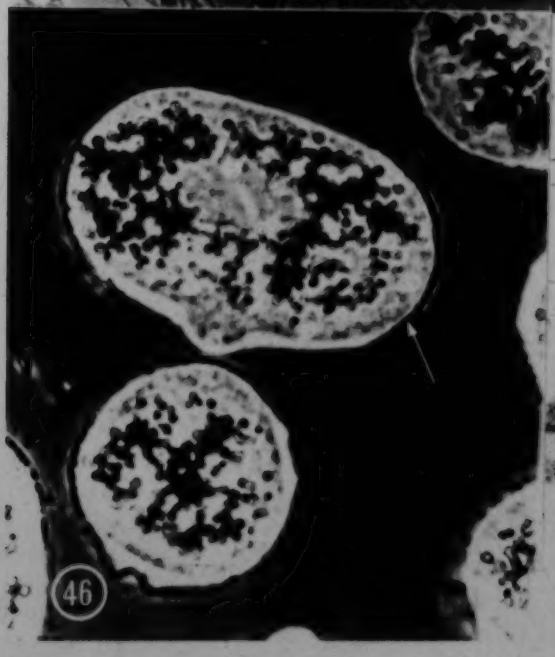

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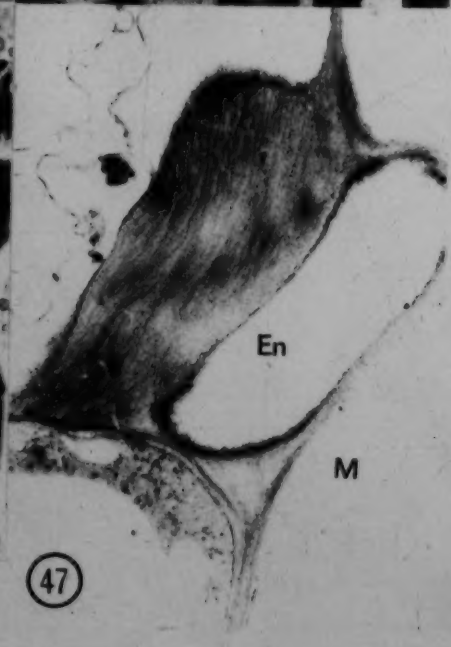

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Fig. 48: Electron micrograph of Lens mature pollen cytoplasm. Mitochondria and numerous vesicles are present in the outer cytoplasm. x6000.

Fig. 49: Electron micrograph of Pisum mature pollen cytoplasm in aperture. Mitochondria, dictyosomes, vacuoles and electron-dense deposits are present. x7800.

Fig. 50: Electron micrograph of Lens mature pollen cytoplasm - inner zone. Vacuoles and less electron-dense bodies are surrounded by rough endoplasmic reticulum with less electron-dense material sandwiched between the two strands. x22530.

Fig. 51: Electron micrograph of Lens exine; beneath the nexine 2 layer is the narrow intine. Above the nexine 1 layer are the baculae and tectum. Interbacular lipid deposits (arrow) are present. x12670.

V
Vs
48
Vs
49
RER
Vs
50
TMR
To
N2
51

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APPENDIX I

Fixation and embedding schedule

Steps 1-6 were carried out at 5C.

1. Anthers were fixed 3 hrs. in 3% glutaraldehyde in phosphate buffer at pH 6.8.
2. Washed with phosphate buffer at pH 6.8 - 4 changes each of 30 min.
3. Post-fixed 2 hrs. in 2% osmium tetroxide in phosphate buffer at pH 6.8.
4. Washed 1 hr. in phosphate buffer at pH 6.8; some tissues not washed.
5. Dehydrated in ethanol series, 10% increments at 30-40 min./increment.
6. Left in 100% ethanol overnight.

Steps 7-10 were carried out at 20C.

7. Transfered to fresh 100% ethanol for 1 hr.
8. Placed for 1 hr. in 1:1 mixture of 100% ethanol and propylene oxide.
9. Three changes of 100% propylene oxide in 2 hrs.
10. Infiltrated in Spurr's resin and propylene oxide series.

Resin made: Vinylcyclohexene dioxide 10gm.
Diglycidyl ether of polypropyleneglycol 6.5gm.
Nonenyl succinic anhydride 26gm.
Dimethylaminoethanol 0.4gm.

Above mixture produces moderately soft block.

(a) At first increments of 10% were used, 45 min./solution.

(b) Later it was found that 20% increments could also be used without penetration problems, 1½ hrs./solution.

(c) For mature pollen infiltration time was increased in the 20% series, 8-12 hrs./solution.

(d) Material was left uncapped in the 80% solution overnight so that remaining propylene oxide evaporated off.

(e) Placed in fresh resin for 8 hrs.

11. Polymerized overnight in Beem capsules at 60C.

APPENDIX II

Staining procedures

Aceto-carmine (adapted from Darlington and La Cour 1960).

Chromosomes did not stain readily. Neither mordanting nor boiling in stain solution improved the results.

Aniline Blue Black (adapted from Fisher 1968).

The same procedure was used as outlined by Fisher (1968) except that after washing slides were re-dried on the hotplate and mounted in Clearmount. This worked as well as air drying and mounting in glycerol in 5% acetic acid.

Aniline Blue Fluorescence (adapted from Jensen 1962).

1. Plastic embedded sections were stained for 2 hrs. to overnight in .005% aniline blue in 0.15M phosphate buffer at pH 9.5.
2. Mounted in staining solution.
3. Viewed under UV fluorescence on the photomicroscope using; excitation filters I and IV which transmitted light wavelengths between 300-500nm, barrier filters 47 and 53 which transmitted light wavelengths greater than 450nm.

Chlorine Sulphite Test (adapted from Siegl 1953 and Jensen 1962).

1. Fresh tissue used without pretreatment.
2. Tissue placed in Chlorox for 5 min.
3. Tissue transfered to 1% sodium sulphite solution.

IKI Reaction (adapted from Johansen 1940 and Jensen 1962).

1. Fresh material: Sections were mounted in the staining solution. After 3-5 min. a reaction should have occurred.
2. Plastic embedded: (a) Tissue stained in IKI 3 min. at 50C.
   (b) Rinsed in distilled water.
   (c) Slide was dried at 50C.
   (d) Sections mounted in Clearmount.

Periodic acid Schiff's Reaction (adapted from McManus 1948).

1. Plastic embedded tissue was placed in 5% periodic acid at 20C for 10-25 min.
2. Washed in running water 2-10 min.
3. Placed in Schiff's reagent 20-45 min.
4. Washed in running water 10-30 min.
5. Water-mounted slide viewed under microscope.

Toluidine blue (adapted from Trump et al. 1961).

1. Plastic embedded tissue was stained with 1% aq. Toluidine blue in 1% aq. borax at 60C for 10 min.
2. Tissue rinsed in distilled water.
3. Slide dried at 60C.
4. Sections mounted in Clearmount.

Uranyl acetate (adapted from Gibbons and Grimstone 1960).

Thin sections were stained on grids for 4 min. in equal parts aqueous saturated uranyl acetate and either 100% ethanol or 100% methanol. Each of the 2 alcohols was equally effective.